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Jacques Monod, Theory and Application of the Technique of Continuous Culture (Pasteur Institute, Microbial Physiology Branch)

Translator: R.T. Hyde, May 1952

I. INTRODUCTION

To transplant a bacterial culture is, in the present meaning, to dilute a small volume of culture in a large volume of new medium. This operation introduces a discontinuity in growth, and into the experiment an element of uncertainty that is well known to bacteriologists. The discontinuity and uncertainty will be reduced to the extent that transfers are made more frequently and performed at smaller dilutions [e.g., with less dilution involved.—Tr]. At the limit one would have a culture maintained by continuous dilution so calculated as to compensate exactly for cell growth. A culture thus maintained would grow indefinitely, at a constant rate, under constant conditions. The discontinuity would disappear and likewise the element of uncertainty that it introduces. It is evident that through the constancy of conditions in the medium, the rate of growth, and therefore the physiological state of the cells, such a culture would be an extremely favorable ~~experimental~~ object for experiment.

The researches pursued in this laboratory have brought us to put this principle to work (Monod, Torriani, and Doudoroff, 1950)[27] and to study the properties of continuous cultures. One will see by what follows, I hope, that the interest in this technique is not limited to the obtaining of permanent and stable cultures. Continuous cultures constitute, under certain conditions, systems in equilibrium, such that the study of a phenomenon ~~or function of time could there be often substituted~~ as a function of time could there be often substituted for by its measure of [OR proportionally in—Tr] the state of equilibrium, which would present great advantages, theoretical as well as practical.

I shall try here to present the general properties of these continuous systems, and to indicate the means of using them in different types of experiments. This is only a first approximation. The problem deserves to be treated, from the theoretical point of view, in a more rigorous and profound manner than I know how to do. As for the technique of realization, one will find here only the description of a very primitive apparatus, of which the sole merit is simplicity.

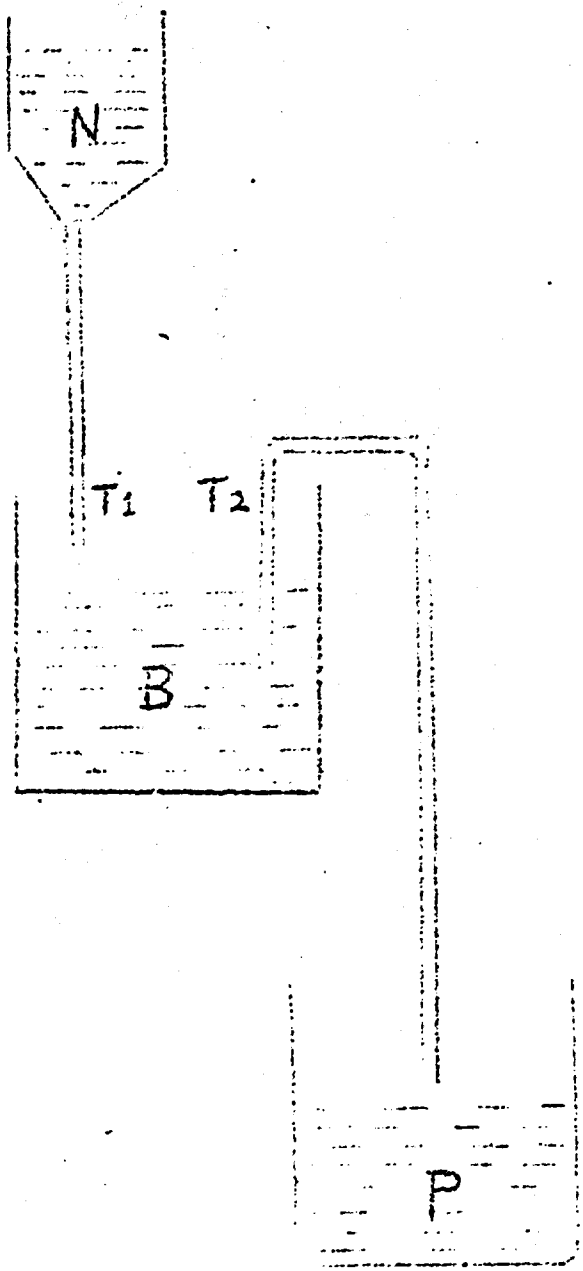

FIGURE 1. Diagram of an apparatus for continuous culture.

II. THEORY

A. CONTINUOUS EXPONENTIAL GROWTH: CONDITIONS OF EQUILIBRIUM

Let us consider a container, B, containing a given volume, V_B, of bacterial culture. Let us suppose that, conditions of the medium being favorable, this culture develops at a constant rate. Suppose that new medium, in reserve in a reservoir, R, is fed continuously into the container, B, by a tube ad hoc (T_1), while, by some device, an equal quantity of medium is withdrawn at the same instant by a second tube (T_2) leading to a second receptacle (P). Suppose that the bacteria falling into the receptacle P cease immediately to multiply (because they are congealed /OR frozen—Tr/, or because the receptacle P contains an antiseptic or bacteriostatic substance). Suppose finally that despite the new medium contantly admitted into the culture, the homogeneity of the bacterial suspension and of the nutrient substances dissolved is assured by an effective stirring of the liquid in the container B. This stirring is supposed to assure likewise the equilibrium between the liquid medium and the gaseous atmosphere of the container.

Let x_B be the bacterial mass contained in the container B,
x_P the bacterial mass contained in the receptacle P, and
x_T be the total bacterial mass.
One has by definition:

$$x_B = x_T - x_P$$

and in deriving with reference to time:

$$\frac{dx_B}{dt} = \frac{dx_T}{dt} - \frac{dx_P}{dt} \qquad (1)$$

It being given that only the bacteria contained in B are multiplying, one sees that:

$$\frac{dx_T}{dt} = \mu x_B \qquad (2)$$

"Malzerian rate of growth."

μ is a constant that we will call "natural-Napierian growth" (1). The growth of the mass bacterial mass in P is at each instant proportional to x_B and to the flow rate of the system. If we define this flow rate (D) as the ratio of the volume flowing per unit of time to the volume V_B, we can write:

$$\frac{dx_P}{dt} = D x_B \qquad (3)$$

1. The rate of growth is generally defined as the number of divisions or [illegible] per unit of time. One considers then Napierian rate of $\mu = \frac{\log 2}{t_g}$.

In combining (1), (2), and (3) one ~~minimum~~ obtains:

$$\frac{d x_B}{dt} = (\mu - D) x_B \qquad (4)$$

and in integrating:

$$Log_e \frac{x_B}{x_o} = (\mu - D) t \qquad (5)$$

x_0 is here a constant expressing the bacterial mass at the time t 0.

If we consider any interval of time, $t_2 - t_1$, and the corresponding growth, $x_2 - x_1$, of the bacterial mass in B, we can write:

$$\frac{Log_e x_2 - Log_e x_1}{t_2 - t_1} + D = \mu \qquad (5 \text{ again})$$

This equation permits calculation of the rate of growth, given the growth of the culture and the rate of dilution.

The system is in equilibrium when $\mu = D$. In this condition, the bacterial mass or, what amounts to the same, the density of the culture in B is constant, while the "product" accumulates in P proportionally to time.

If one arranges a means of regulating the flow rate, it is then possible, in principle, to maintain the culture indefinitely at the greatest density compatible with maximum rate of growth, while multiplication continues at a rate corresponding to the exponential phase.

D. CONTINUOUS GROWTH AT A LIMITED RATE

The growth of a culture in a medium not renewed modifies the composition of the medium and thus creates conditions that retard and, finally, arrest the growth. In the constantly renewed medium of the container B, this does not occur if the rate of dilution balances exactly the ~~entire~~ maximum rate of growth. If ever the rate of dilution is ~~less~~ than the rate of growth, it will work out otherwise. The density of the culture in B will increase at an "apparent" rate equal to $\mu - D$, while the conditions of the medium remain optimal. Sooner or later the contribution of new medium will no longer suffice to maintain these conditions for an increased population, with the result that the rate of growth will diminish. It is clear that it will attain thus, necessarily, the value of equilibrium ($\mu = D$), but that it will not fall appreciably below this value, for then the population will decrease and whatever surplus /of new medium—Tr/ is excess will tend to restore the original conditions, consequently increasing the rate of growth. This will thus adjust itself automatically to the value of equilibrium and remain there indefinitely.

These conditions of operation by which the system tends toward a stable equilibrium are particularly interesting, and we shall try to define them more precisely. Let us consider a culture developing in a medium of definite composition, and such that the sole "limiting factor" of growth is the concentration of one of the essential elements, for example the element carbon. One knows (Cf. Monod, 1942-1949)/1/ that in such a medium (not renewed) the total growth (2) is proportional to the initial concentration of the element carbon. One knows also that the rate of growth varies with the concentration of the element carbon according to a law fairly well expressed by the hyperbolic relation:

$$\mu = \mu_0 \frac{S}{S_K + S} \qquad (6)$$

in which u is the rate of growth corresponding to a concentration S of the limiting element, u_0 the maximum rate of growth, which provides when S is great, S_K, a constant, characteristic of the organism and of the substance being considered.

One knows finally that, in most of the known cases, the value of the constant of "affinity" S_K is very small compared to the values of the concentration S permitting an amply abundant development of the cultures. This is to say that the rate of growth is, in practice, independent of the concentration of the element carbon except when it is exceedingly small.

Let us consider now a culture developing in the container B (fig. 1) fed by a reservoir containing a medium of such composition that the concentration of the element carbon constitutes the sole limiting factor of the growth. Let S_0 be the concentration of the limiting element in the new medium; S, the concentration of this element in B; R, the constant of yield (Cf. Monod, loc cit). One may write:

$$\frac{dS}{dt} = D(S_0 - S) - \frac{1}{R}\frac{d\chi_{gr}}{dt} \qquad (7)$$

In effect, first, the concentration S of the element in B tends to approach the concentration S_0 in the new medium more quickly as the difference is greater and the flow rate more rapid; second, the concentration S tends to diminish more rapidly as the growth of the total bacterial mass is more rapid. This second term

$$\left(-\frac{1}{R}\frac{d\chi_{gr}}{dt}\right)$$

expresses the hypothesis that the yield of the growth is constant, independent of its rate (3). In substituting /See p 5—Tr/

2. Difference between the initial density and the maximum density. The density of the culture is defined as the dry weight of bacterial substance per unit of volume.

3. See on this subject Monod (1942)/1/, Teissier (1942)/3/, and below, p 393 /o.c., p of this translation—Tr/.

. . .In ~~minimizing~~ replacing

$$\frac{dx_T}{dt} \qquad \frac{dx_T}{dt}$$

in the equation (7) by its value drawn from equation (2), one obtains:

$$\frac{dS}{dt} = D(S_0 - S) - \frac{x_B}{R}\mu$$

and in replacing μ by its value, given by (6):

$$\frac{dS}{dt} = D(S_0 - S) - \frac{x_B}{R}\mu_0 \frac{S}{S_K + S} \qquad (8)$$

A culture cannot be said to be in equilibrium unless S and x_B are constants, that is to say when one has at once:

$$\frac{dS}{dt} = 0$$

$$\frac{dx_B}{dt} = 0$$

According to /From—Tr/ equations (4), (6), and (8), these two conditions can be written respectively:

$$D(S_0 - S) = \frac{\mu_B}{R}\mu_0 \frac{S}{S_K + S} \qquad (9)$$

and

$$\mu_0 \frac{S}{S_K + S} = D \qquad (10)$$

It is evident that equilibrium is not realizable if $D > \mu_0$ ~~max~~ since the equation (10) would not be verified for any value of S or of D. In return, a stable equilibrium is necessarily attained if $D < \mu_0$. In effect, if at any moment x_B is inferior to the value ~~max~~ satisfying the equation (9), then

$$\frac{dS}{dt}$$

is positive. S increases, such that

$$\mu_0 \frac{S}{S_K + S} - D$$

takes a positive value, and the culture increases. The opposite is true /lit "The inverse is produced..."—Tr/ if x_B is superior to the value of equilibrium. Likewise, if S is greater than the value satisfying the equation (10), then

$$\frac{dx_B}{dt}$$

is positive /in?/ equation (4), the culture grows, so that the equality (9) is no longer satisfied, and

$$\frac{dS}{dt}$$

becomes negative. The reverse is produced if S is inferior to the value satisfying the equation (10). If, the equilibrium being obtained, one modifies the flow rate (while maintaining it below μ_0), the system evolves, for the same reasons, toward a new equilibrium: the bacterial density and the concentration of the limiting element stabilize themselves at new values, such that the rate of growth is again equal to the rate of dilution. The experimenter thus has thus a means of modifying at his will the rate of growth and of adjusting it to any value less than μ_0.

One sees, from (10), that at equilibrium the equation (9) is simplified:

$$\chi_B = R(S_0 - S) \qquad \text{(9 also)}$$

Furthermore, as the stable equilibrium requires

$$D = \mu_0 \frac{S}{S_K + S} < \mu_0$$

Since

SF, on the other hand, the value of the fraction

$$\frac{S}{S_K + S}$$

is practically independent of S as long as S is of an order of magnitude greater than S_K; because finally the values experimentally determined (see mention above) for S_K are very small, equilibrium will be attained only for weak values of S.

This observation is important since, in practice, the values chosen for the initial concentration, S_0, will be almost invariably much greater than the values of equilibrium of S. At equilibrium, $S_0 - S$ will then be very little different from S_0, and the equation (9 also) is simplified further to

$$\chi_B = RS_0$$

In other words, everything occurs as if, in spite of the different concentrations existing in the bottle, the limiting element were entirely consumed by the bacteria. Let us clarify this conclusion by an example. For E. coli growing in a chemically defined medium, with glucose as the limiting element, the constant S_K is of the order of 10^{-5} (Monod 1942). But, the concentration of glucose that would give a "good culture" in the eyes of a bacteriologist would be from 10^{-3} to 5×10^{-3}, or 100 to 500 times greater. With $D \leq 0.5\ \mu_0$, the equation (10) would be:

$$\mu_0$$

$$0.5\,\mu_0 = \mu_0 \frac{S}{10^{-5}+S}$$

where S 10^{-5}. The equation (9 also) would give us then, with S 2.10^{-3},

$$x_B = R(2.10^{-3} - 10^{-5})$$

and one sees that x_B would differ by only 1,200 from the maximum value corresponding to the integral utilization of the element carbon. With D $0.9\mu_0$, the density of the culture, at equilibrium, would not be less than five percent (/5 p. 100/) less than the maximum value. / would be inferior by only five percent of the maximum value.

It appears then that under these conditions, not only is the system self-regulating, but also the rate of growth may be fixed at any desired value (below about $0.9\mu_0$) and modified even in the course of an experiment without meanwhile subjecting the density of the culture to appreciable variations. These are especially the singular properties of a "self-regulating system" which establish interest in continuous cultures; in the paragraphs that follow one will see how these properties can be used with profit for the study of some typical problems of microbial physiology.

C. APPLICATION TO SOME PROBLEMS OF MICROBIAL PHYSIOLOGY

Relation between Rate of Growth and the Concentration of a Limiting Element

The study of this relation constitutes evidently one of the most immediate applications of the technique of continuous culture. When for such a study one utilizes cultures in unrenewed medium, one encounters grave difficulties that lead to weak values of the constants of affinity /relationship/: in most cases, the concentrations of the limiting element giving visible cultures are found in the zone of saturation. The problem consists then of maintaining the concentration of the limiting element at constant and very weak values. But, that is the result obtained automatically with a continuous culture when the flow rate is less than the maximum growth rate, that is to say in a self-regulated system.

It is not necessary to insist on the evident fact that the employment of this technique is not justified unless the constitution of the medium is such that the element being studied constitutes indeed the only limiting factor of the growth (Cf. on this subject, Monod, 1942, p. 35). This granted, it is not therefore inevitably necessary that the general form of the relation be known. It is necessary and it suffices that a stable equilibrium be obtained for which one may write:

$$\mu = D = f(S)$$

Then, a stable equilibrium cannot fail to be obtained, if only the solution presents the general form of a saturation curve, which, a priori, seems needed to be always the case /e.g., seems necessarily to be?—Tr/. The direct dosage of the limiting element, in the liquid of the culture, permits then establishment of the form of the relation $\mu = \phi(S)$.

It is no longer necessary, in order that the technique be applicable, that the variations of x_3 (density of the culture) with the rate of dilution be negligible. In fact, the only limit to the application of this technique will be the precision of the chemical dosage. One can, in principle, avoid the direct dosage of S, by deducing its value from the relation (9 also):

$$x_B = R\,(S_o - S)$$

But since the variations of x_B at different equilibriums will be always weak (or even insensible /undetectable?/, the errors would be considerable. Furthermore, as we shall see, the relation (9 also) represents an approximation that can be faulty in certain cases.

Growth Yield

In effect, to consider the equation (9 also) as exact, return to admit the hypothesis expressed by the second term of the equation (7) that the yield of growth is independent of the rate of growth. When /If/ this hypothesis is acceptable, the equation (9 also) permits determination of the constant of yield, R. The dosage of the limiting element at equilibrium is useless. In principle, it is sufficient to determine x_B for two or three values of S_o. ~~Thereby~~ That is not a particularly interesting application of the method, since, to the ~~same~~ same extent that the hypothesis of independence is correct, one could use equally well the usual techniques. One knows that this hypothesis verifies ~~itself~~ itself /is verified/ with precision when growth is limited by ~~carbon~~ /lability/ of the element carbon (Cf. Monod, loc cit). It is probable that it represents a good approximation in most cases, but that it ceases to be exact beyond certain limits, or for certain elements. For example, any communal nutrient affected by an appreciable "coefficient of maintenance /supply/" cannot be /"will not know how to be"/ independent of the rate of growth. If one lacks data on this point, it is partly because one does not have available an adequate technique. The "continuous cultures" give one the ability to vary the rate of growth without having to modify ~~him~~ either the temperature or the composition of the medium (if that is not the concentration of one element), offering a solution to this experimental problem. If we admit that the yield, R, is a function, $\phi(\mu)$, of the rate of growth, the equation (9 also) becomes:

$$\frac{x_B}{S_o - S} = \phi(\mu) = \phi(D) \qquad (9\text{-c})$$

The determination of S and of x_1 for different rates of dilution permits establishment of the form of the relation between the rate of growth and the yield.

Meanwhile, it must not be forgotten, this method is not justified unless the yield is independent of the concentration of the element. This is a second "hypothesis of independence" which could prove faulty when the concentration of the element varies much, or when it becomes very small, which is precisely the case for the limiting element in equilibrium. One imagines, in effect, that a substance metabolized by two distinct enzymatic systems with very different constants of affinity could not give the same yields at strong and at weak concentration. The solution of this difficulty consists in composing the medium so that the limiting factor will be a nutrient source other than that of which one wishes to determine the yield. The equation (7), hence the equation (9-c), is not rendered less valid by that. It is still necessary, however, that the concentration S_0 of the element studied not be chosen too large, in order that S_0-S may be measurable with precision, nor too small, so that S remains large enough so that its variations may be considered as without effect.

The variations of yield as a function of the concentration of one element can have, in certain cases, a particular interest. Here again the device is applicable of limiting the rate of growth by an element other than that of which the yield is being studied. D being maintained constant, one determines S by dosages, for different values of S_0.

One knows finally that the yield of bacterial growth is a function of the temperature (Cf. Monod, 1942, p 106). But it is not possible, by the usual techniques, to study this effect independently of the concomitant variations of the growth rate. The continuous mmk culture at a limited rate permits attainment of this result in certain conditions. Let us suppose that, by regulation of the flow rate, the rate of growth of a culture is fixed at a value slightly less than the maximum rate corresponding to a given temperature. Suppose that this temperature is fairly far from the optimum. This culture could be brought to any temperature at which the maximum rate is superior to /greater than/ the fixed flow rate, without modifying the growth rate. It is evident, however, that the increase of temperature is manifested, in fact such conditions, by a variation (in general a diminution) of the concentration of equilibrium of the limiting element. One must eventually take into account the effect of dilution discussed in the preceding paragraphs, and to avoid it by such means that the limiting element will not be that for which one seeks to determine the yield as a function of temperature.

The possibility of dissociating, in a certain measure, two physiological phenomena, both functions of temperature, constitutes no doubt one of the more interesting applications of the method. We shall return to this topic presently.

Rates of Synthesis

The study of the kinetics of a specific process of synthesis, such as the formation of an enzyme or other cellular constituent, involves grave difficulties, and of all sorts, most inherent in the phenomenon itself. Certain of these difficulties however arise from the techniques of culture. The suspensions termed "nonproliferating" cannot be used if it [illegible] concerns a phenomenon bound, even indirectly, to growth. Besides, the properties of these suspensions, which contain a variable, generally undetermined number of nonviable cells, change rapidly with time. A culture in process of growth cannot be considered as physiologically stable except in the course of the exponential phase, often too brief for the requirements of the experiment. Also the composition of the medium is modified very rapidly during this phase. Finally, the continual variation of the bacterial density during the experiment introduces an additional difficulty. The use of continuous cultures that one can maintain indefinitely in a constant medium, is then indeed indicated.

Continuous cultures present for this type of experiment other remarkable advantages. In the first place, to measure the rate of a reaction of synthesis in a continuous culture returns to determination of a state of equilibrium, instead of measuring at successive intervals the accumulation of a substance. In the second place, the control of the rate of growth permits study of the degree of dependence (or of independence) of the reaction of synthesis under consideration, with respect to the processes of synthesis in combination /e.g., all together—Tr/. In sum, it permits distinguishing, in a certain measure, the effects of an active agent simultaneously on the phenomenon being studied and on others that could mask it. In order to state precisely the properties of these systems, we shall consider now a few theoretical models of reactions of synthesis.

Let us assume a continuous culture, engaged in growing in conditions of self-regulated operation. Assume a cellular constituent, Z, an enzyme for example, synthesized by the bacteria in the course of growth /or, in the growth process—Tr/. Let Z_B be the quantity of Z in the container B, Z_P the quantity of Z in the receptacle P, and Z_T the total quantity.

One can write:

$$\frac{dZ_B}{dt} = \frac{dZ_T}{dt} - \frac{dZ_P}{dt} \qquad (11)$$

The growth of Z in the receptacle P is given by:

$$\frac{dZ_P}{dt} = Z_B D \qquad (12)$$

D representing, as above, the rate of dilution.

To have a complete system of equations [homologues of the equations (1), (2), and (3)], it remains to express the growth of Z_T as a function of the conditions of the medium, of Z_B, eventually of the rate of growth, of the temperature, etc., that is to establish a hypothesis on the mechanism or the nature of the processes of synthesis by which the substance Z is elaborated. This equation, which we shall call "hypothetical" can, according to the mechanisms considered, take very different forms. However, setting aside certain cases that it would be necessary to qualify by pathologies, the hypothetical equation would have to express the fact that the concentration of any cellular constituent cannot exceed a certain limit. Let X be that limit, which is expressed for example as [the? the? —Tr/ fraction] of the bacterial mass x_B contained in the container B. The general hypothetical equation will be of the form:

$$\frac{dZ_T}{dt} = (X - Z_B)\,\psi \qquad (13)$$

in which ψ represents the hypothetical function, so called. In comparing the equations (11), (12), and (13), one sees that the system tends necessarily toward an equilibrium, since

$$\frac{dZ_T}{dt}$$

diminishes while

$$\frac{dZ_P}{dt}$$

increases when Z_B increases, and inversely:

$$\frac{dZ_B}{dt}$$

tends then always toward zero. This is with the condition that ψ will not take infinite values, a hypothesis that one can exclude *a priori*. Let Z_∞ be the value of Z_B at equilibrium. The determination of Z_∞ gives a measure of the speed [rate] of the reaction of synthesis in the chosen conditions, since at equilibrium one can write, from (11) and (12):

$$\frac{dZ_T}{dt} = \frac{dZ_B}{dt} = Z_\infty D$$

Let us see now how Z_∞ varies according to the form of the function ψ that is to say according to the hypothesis based on the mechanism of the reaction (or of the system of reactions) of synthesis. It is clear that all sorts of hypothetical functions could be visualized according to the variables and the phenomena considered. We shall confine ourselves here to a few simple and typical cases, of a nature to [illegible] bring to light the properties of the system.

Let us suppose first that one wishes to study the effect on the synthesis of Z_T of a substance present in the medium. Let S be the concentration of this substance. Let us make, on the mechanism of action of this substance, the simplest hypothesis possible: that the speed /rate, rapidity—Tr/ of the reaction of synthesis is proportional to the concentration S. The function ψ becomes then:

$$\psi = KS$$

K being a constant of proportionality.

The hypothetical equation (13) is written then:

$$\frac{dZ_T}{dt} = (X - Z_B)KS \qquad (13a)$$

Since at equilibrium one has:

$$\frac{dZ_T}{dt} = \frac{dZ_P}{dt} = Z_E D$$

and, by definition,

$$Z_B = Z_E$$

One can write in replacing

$$\frac{dZt}{dt}$$

and Z_B by their values:

$$Z_E D = (X - Z_E)KS$$

whence:

$$Z_E = X\frac{KS}{D + KS} \qquad (4)$$

In this case, as one perceives, Z_E would be a hyperbolic function of D and of S. Its value would tend toward X when S becomes large, or when D tends toward 0. Z_E would be null only for $D = \infty$ or $S = 0$. The figurative curves as a function of S and of D are given by the figures 2 and 3. Let us note that in this calculation we admit implicitely that S is independent of D. That is to say that the "active" substance of which the effect is to be determined cannot be the limiting element. There is no difficulty there, however. It is however necessary to take care that the concentration S at equilibrium is not equal to the concentration S_0 in the new medium, if the active substance is metabolised at an appreciable rate. Keeping account of these observations, the experimental verification of the hypothesis as well as the determination of the value of the constants does not offer difficulty of principle.

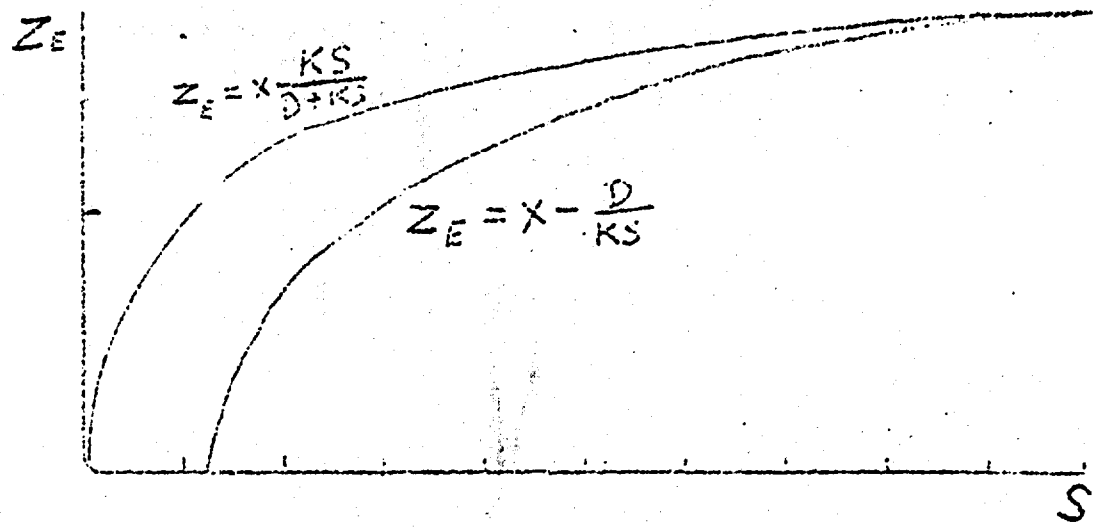

FIGURE 2. Theoretical curves expressing the variation of the concentration of equilibrium of one constituent, Z_E, as a function of the concentration of the inductor, S. Above: ~~[illegible]~~ reaction (Equation A). Below: Self-catalytic reaction (Equation B). [Above, upper; below, lower curve.—Tr.]

Non-self-catalytic ["nonautocatalytic"]

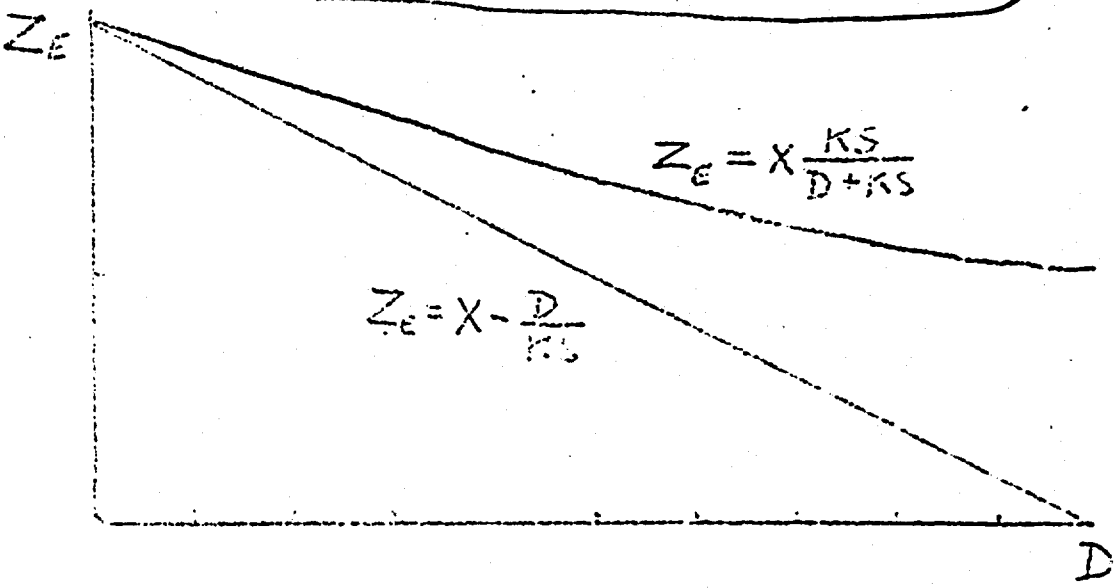

FIGURE 3. Theoretical curves expressing the variation of the concentration of equilibrium of the constituent E as a function of the rate of dilution, D.
Above (upper): ~~[illegible]~~ Non-self-catalytic reaction (Equation A).
Below (lower): Self-catalytic reaction (Equation B).

TRANSLATOR'S NOTE: Caution: These curves have been merely sketched, not worked. See original French text, p 45.

Let us suppose now that, keeping the same hypothesis as to the effect of the activating substance, we establish the supplementary hypothesis that the reaction is autocatalytic, stated otherwise that its rate is at each instant proportional to the concentration of the substance Z in the cells. The hypothetical equation becomes:

$$\frac{dZ_T}{dt} = (X - Z_B) \qquad (13b)$$

The same reasoning as above leads to the following solution for the state of equilibrium:

$$Z_E = X - \frac{D}{KS} \qquad (B)$$

The figurative curves of Z_E as a function of D and of S, according to equation (B) are given by figures 2 and 3. One sees that these curves are very different from those derived from the first hypothesis /noncatalytic reaction, equation (A)/. In particular, Z_E is a linear function of D, and is cancelled for a finite value of the variable. In other words, when the flow rate increases beyond certain values, the substance Z is no longer formed. Likewise, Z_E becomes null when the concentration of the ~~substance~~ active substance falls below a limited value. Supposing that the experiment cannot be done for the critical values of D and of S, extrapolation should permit distinction between the experimental results that would verify either equation A or equation B. The measure of Z_E for different values of flow rate and of the concentration of the active substance should then permit determination whether the process of synthesis of the constituent Z is or is not given from /associated with, or a result of?—Tr/ the properties of an autocatalytic reaction.

The hypotheses expressed by the equations (13a) and (13b) suppose implicitly the independence between the rate of growth and the rate of the reaction of synthesis. If one supposes, on the contrary, that the rate of this reaction is directly proportional to the rate of growth, μ, the terms D and u disappear from the solution, since in a self-regulated system D u. The equation A would become:

$$Z_E = X \frac{KS}{C + KS} \qquad (A')$$

and the equation B

$$Z_E = X - \frac{C}{KS} \qquad (B')$$

C represents a constant. One sees that in this case Z_E would become independent of D, whatever may be otherwise ~~the~~ the form of the

hypothetical function. The determination of S_κ as a function of D would then permit the eventual establishment of the limits of validity of the "hypothesis of independence".

We have supposed thus far, for greater simplicity, that the speed /or rate—Tr/* of the reaction of synthesis was directly proportional to the concentration of the "active substance", S. One can visualize also the case, more probable, where the reaction would be enzymatic in nature. Its rate would no longer then be proportional to S, but to a function of S that would express the phenomenon of saturation characteristic of enzymatic reactions. If, for example, one adopts, for this function, the form of the equation of Michaelis:

$$v = V \frac{S}{S_\varepsilon + S}$$

in which V represents the maximum rate and S_κ the constant of affinity, the equations (A) and (B) become respectively:

$$Z_\varepsilon = VX \frac{S}{DS_\kappa + DS + VS} \qquad (A'')$$

and

$$Z_\varepsilon = X - \frac{DS_\kappa + DS}{VS} \qquad (B'')$$

It will be seen without difficulty that the equations (A'') and (B'') lead to experimental conjectures that cannot be confused with one another, nor with the assumptions of the homologous equations (A) and (B). The experiment based on these conjectures could then in principle separate these different hypotheses.

If, instead of choosing as a variable the concentration of a substance supposed "activating", we had introduced a constant of rate, supposedly a function of some independent variable, the results would be the same. Such an independent variable would be, for example, the temperature. We have just seen that the conditions of a self-regulating system /or process—Tr/ could be so fixed that the rate of growth would be independent of the temperature during a sufficiently large interval. One sees now how variations in temperature, at a constant rate, could be used for analysis of the mechanism of a reaction of synthesis.

One can visualize also many other forms of the hypothetical equation, intended to express all sorts of mechanisms. But it is a matter here of bringing out only the principles. The examples that we have just discussed suffice to show how the measure of the concentration of a cellular constituent in a continuous culture brought to equilibrium permits study, as functions of diverse variables, thus of the properties of the reaction of synthesis.

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* The author uses taux ("rate") with reference to croissance (growth"), and vitesse ("speed" or "rapidity") with reference to reaction de synthese ("reaction of synthesis"). Perhaps vitesse implies a completed process and taux a continuing one, or perhaps the terms are synonymous?—Translator's note.

The advantages, however considerable they may be in theory as well as in practice, of this method of equilibrium, should not make us forget that a continuous culture lends itself equally well, and much better than a normal culture, to measurement of the accumulation (or of the disappearance) of a cellular constituent as a function of time. In order for the employment of this second method to be justified, it is necessary and it suffices that the conditions of culture (rate of growth, flow rate, composition of medium) do not vary in the course of the test. This acquired, one has at disposal there a second means of verifying experimentally the assumptions of the hypothetical equation. In replacing

$$\frac{dZ_T}{dt} \text{ and } \frac{dZ_P}{dt}$$

in the equation (11) by their values drawn from (12) and the hypothetical equation (13) one obtains:

$$\frac{dZ_B}{dt} = K(X - Z_B)\Psi - Z_B D$$

or in integral form:

$$\int \frac{dZ_B}{KX\Psi - (K\Psi + D)Z_B} = \int dt + Cte$$

The integration gives Z_B as a function of time. For the verifying experiment to be significant, it is evidently necessary that at zero time the concentration Z_B be different from the concentration of equilibrium Z_E corresponding to the chosen conditions. But, and this is very important, if the equilibrium is reversible, it is immaterial whether Z_B at the initial time is less than or greater than Z_E. Practically, the experiment will consist first of an equilibrium obtained in the given conditions, then modification of the conditions of equilibrium, and then measurement of Z_B at adequate intervals until the new equilibrium is realized. In principle, one can impose on a single culture an indefinite succession of these ruptures of equilibrium. There is no need to ~~emphasize~~ /[illegible] it/ is useless to insist on..."—Ty/ the wealth of experimental combinations offered by this technique.

Rate of Mutation

One can only mention here the possible application of the technique of continuous culture to the study of bacterial mutations. A real discussion of this problem would carry us too far afield. One knows that the theories relative to the selection of mutant forms in bacterial populations are not in general of value except for cultures [illegible] that are [illegible] at a constant rate. Furthermore, the hypothesis is almost always admitted, but has never been demonstrated, that the probability of mutation is proportional to the rate of growth. The verification of this fundamental hypothesis seems possible with continuous cultures at limited rate, as well as the determination, at a constant rate of growth, of the coefficients of temperature of the /[illegible] the?—Ty/ frequencies of mutation.

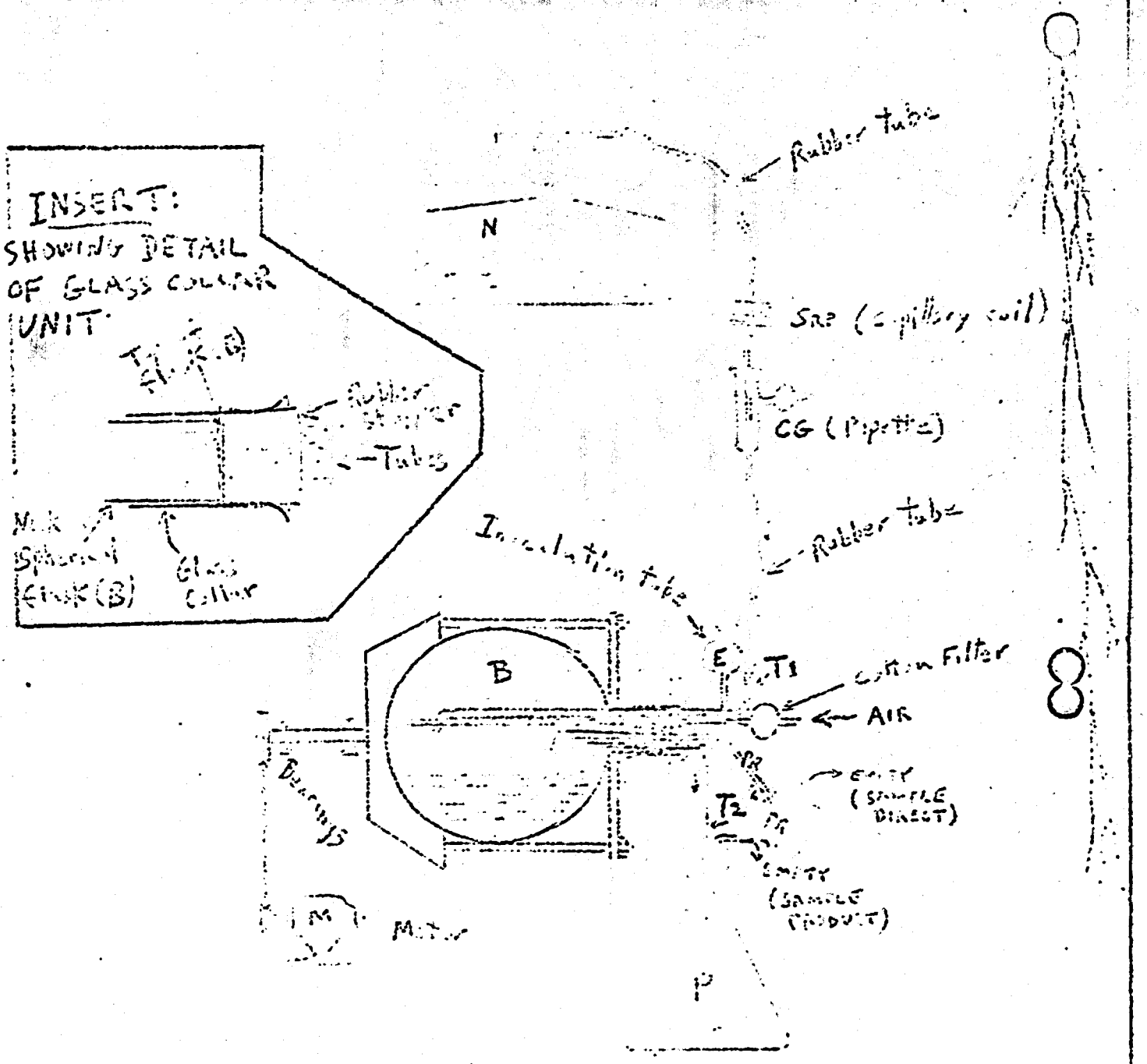
III. REALIZATION

A. EQUIPMENT

The preceding theoretical developments are based on quite rigorous definitions of the conditions of culture. It would be vain to seek to apply the theory if the experimental conditions deviated too sensibly from these definitions. Various very different types of apparatus can be imagined that undoubtedly would permit realization of conditions sufficiently close to the theoretical exigencies. I shall describe here only one. This assembly is shown schematically by Figure 4.

The most important condition is homogeneity of the culture, without which the notion of "concentration of equilibrium" of the limiting element would be illusory. It is necessary then that the culture be stirred continually /The author appears to mean continuously, but didn't say so.—Tr/, entirely, and rapidly. It is necessary also that equilibrium with the gaseous phase be assured, which requires that the ratio of the surface to the volume be large. In the assembly chosen, the culture container is a round-bottomed flask, B, fixed and centered on a rotating support. The capacity of the flask is two liters for a culture volume of 100 to 400 cc. /E.g., a 2-liter flask is used for that much culture. The abbreviation cc is given in the text as cm^3. —Tr/ A motor, M, imparts to the flask a speed of rotation of the order of 200 to 400 turns per minute. The stirring obtained is very energetic: at this speed, the dispersion of a drop of dye introduced into the liquid is practically instantaneous. Aeration is assured by the liquid film that covers the greater part of the interior surface of the flask. The best distribution of this liquid film is obtained when the axis of rotation makes an angle of 3 to 4 degrees with the horizontal. /In the illustration, the neck of the flask appears to be tilted slightly upward.—Tr/

The new medium is in reserve in a reservoir /or feeder (nourrice)—Tr/, in this instance a tonin flask, placed [illegible] about 1.50 meters above the liquid level of the spherical flask. The liquid passes through a fine rubber tube and a coil /"serpentine"/ (Serp) formed of a glass capillary tube about 2 meters long, which ends [illegible] in a drop-counting pipette /or dropping tube/. The capillary is chosen of such diameter that the mean flow rate desired is obtained when the end of the pipette is about 1 meter [illegible] below the liquid level of the reservoir. Regulation is accomplished by vertical displacement of the pipette /and, obviously, the capillary to which it is attached—Tr/. One obtains thus a sufficiently stable flow rate without the necessity of maintaining rigorously the constant the level of liquid in the reservoir. The pipette, calibrated by hydrometer (densimeter), permits a precise measure of the flow rate with the aid of a chronograph.



[illegible] Assembly of an apparatus for continuous growth. R, reservoir [illegible], nourished/; SRe, serpentine capillary; CG, drop counter (pipette); B, rotating spherical flask; T_1, inlet tube; E, inoculation tube; EE, sampling tube, sampling flask; T_2, outlet tube (or overflow tube, maintaining liquid level in flask B); P, product; M, motor.

The volume of liquid in B is maintained constant, whatever the flow rate, thanks to a fluid-level /e.g., overflow/ tube (T2) ending in a receptacle P, in which is maintained a sufficient vacuum and where the "product" accumulates. The constant volume does not depend only on the overflow tube T2 but also on the speed of rotation, which determines the thickness, by no means negligible, of the liquid film. An increase in speed not compensated by an adjustment of the tube T2 to the fluid level is translated into an increase in volume, and vice versa. To keep account of this effect one determines the volume by weight after ~~termination~~ testing under the chosen conditions.

The system includes ~~however~~ in addition a tube fitted with a cotton filter through which the air (or any other appropriate gaseous mixture) is led into the spherical flask after having passed through a washing flask containing water. An inoculation tube (E) and a sampling tube (Pr) can usefully complete the device.

The neck of the spherical flask is protected by a ring-shaped piece of glass closed by a rubber stopper through which the tubes pass.

The apparatus is mounted in a cool incubator kept at the desired temperature.

B. CONDUCT OF EXPERIMENTS

The feeding ~~assembly~~ unit—pipette, tubing—is sterilised on the one hand, the spherical flask on the other. The assembly is made sterilly. The spherical flask is filled through the tube E with a "culture of departure" /starting culture—Tr/ of convenient density. The flow rate is primed by creating a temporary depression (low pressure) in the bulb ~~pipette~~ of the pipette. Direct ~~inoculations~~ ~~assembly~~ samples are taken by attaching an empty flask to the end of the tube "Pr".

This said, everything depends on the type of experiment contemplated. Whatever that may be, however, certain precautions will need to be observed: /lit. "must be respected"—Tr/

1. The bacterial density at equilibrium must never be such that the unknown or uncontrolled factors (rate of dissolution of oxygen, for example) will not become limiting. Also it is wise not to exceed 0.3 to 0.5 µg /microgram/ dry weight per cubic centimeter, and to utilize the most diluted cultures whenever it is possible. When one can be content with very dilute cultures (0.05 µg per cc for example), it is easy to compose the media so that the "concentration of equilibrium" of the elements other than the limiting element will be practically the same as in the new medium. This simplifies the experiment, the calculations, and the interpretations.

2. Direct samplings modify the volume in B and consequently the rate of dilution, thus disrupting the equilibrium. It is therefore necessary that they be small enough for the effect to be negligible, or sufficiently spaced to permit the system meanwhile to return to equilibrium. When it is not necessary to effect the sampling at a well defined instant (determination of a state of equilibrium), one can with advantage recover the "product" instead of a direct sample.

3. The conditions in the sample taken change radically and very rapidly: hence the disappearance of the limiting element can be a matter of only a few seconds. Adequate treatment must thus be applied immediately. The most efficacious and the most generally applicable consists of holding the sample on crushed ice.

4. The risks of contamination, with this apparatus, are not negligeable. We have not observed any, however, in the course of numerous series of experiments with a maximum duration of seven or eight hours. The apparatus described was not conceived for a permanent operation.

IV. CONCLUSIONS AND COMMENTS

1. The maintenance of a bacterial culture at a constant density, at a constant rate of growth, in a medium of constant composition, is possible in theory and realizable in practice, thanks to the principle of "continuous dilution at constant volume".

For such a system to be in equilibrium, it is necessary and it suffices that the rate of dilution (ratio of volume of flow per unit of time to volume of culture) be equal to the rate of growth (number of divisions per hour) multiplied by the coefficient 0.69 (Naperian logarithm of 2). This result can be attained in two different ways: (a) by adjusting the rate of dilution so that it balances the maximum rate of growth; this equilibrium is unstable; it can be maintained only by continual adjustments; (b) by letting the culture grow until it becomes limited by one condition (concentration of an essential element), the rate of dilution being fixed at a lower value than that which balances the maximum rate of growth. This equilibrium is stable. The rate of growth is then limited by the concentration of one element, a concentration determined itself by the rate of dilution (self-regulating system).

2. With a continuous culture in a self-regulating system, the experimenter has at his disposal a means of fixing the rate of growth at any desired value below the maximum. In other words, the rate of growth becomes, in a certain measure, an independent variable. That is a valuable experimental resource: it is useless to insist, in this summary, on the numerous possible applications. On the other hand, it is necessary to underscore the danger that will have been foreseen, in the interpretation of experiments, of treating the rate of growth as an unequivocal and abstract variable. The "regulation" of the rate of growth, in a continuous culture, is obtained by the intervention of a limiting factor. Many elements of interpretation depend on the nature of the limiting factor, of its effects on the composition and the metabolism of the bacteria, etc... /Author's punctuation.—Tr/

3. Continuous cultures lend themselves particularly well to the study of the kinetics of processes of synthesis. The composition of a cell in the midst of growth, understood as the relation /or ratio—Tr/ of each cellular constituent to the total mass, tends toward an equilibrium when the rate of growth is constant. The concentration of each cellular constituent, at equilibrium, depends on the constants of speed /rate?/ of the processes of synthesis involved. The determination of the equilibrium obtained (that is to say, the measure of the stable concentration of a cellular constituent), as a function of diverse variables, comes back to determination of the effect of these variables on the speed of the system of reactions by which this constituent is elaborated. This method of equilibrium evidently presents great theoretical and practical advantages. It permits comparison of theoretical models with experimental results under such conditions that many "strange" /or "foreign"—Tr/ factors are cancelled /annulled/ or at least maintained constant. In sum, the technique of continuous culture applied to the study of these difficult problems permits the gaining of a few degrees of freedom in experimentation. But here again the simplification of the experimental problem should not create the illusion that the phenomena themselves are simplified. Were it entirely satisfying, the theoretical model of a process of synthesis would be only a partial representation of a sum of phenomena. There is no fear, besides, that the results will accommodate themselves too easily to naively mechanistic interpretations. These few supplementary degrees of freedom give on the contrary the means of putting theoretical schemes to more formidable and more severe test.

4. The technique of continuous culture undoubtedly would find interesting applications in the analysis of mutability /or mutation?/. But it is necessary to mention in this connection a difficulty that has not been visualized in the theoretical discussion. One has, for this discussion, supposed implicitly that the cultures were genetically homogeneous. Homogeneous more or less in this which concerns their characters or properties under consideration. In experimentation, on the contrary, one cannot neglect *a priori* the factors of selection. The hypothesis of the intervening selection must be considered in all cases, if it be not

eliminated by adequate controls (Monod, Torriani, and Doudoroff, 1950). To know how to take account of it or eliminate it, what controls to use, depends on the problem considered and cannot be discussed here.

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